Exhibit VII

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In Vivo Inhibition of Respiratory Syncytial Virus by Ribavirin†

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Ribavirin reduced the amount of respiratory syncytial virus in nasal turbinates and lung tissues of experimentally infected cotton rats by over 90%. An effect was seen when the drug was given either intraperitoneally or by aerosol; however, the antiviral effect was achieved at much lower doses when delivered by the aerosol route. No animal deaths due to the drug were seen.

Respiratory syncytial virus (RSV) is a major cause of pneumonia and bronchiolitis in infants, in young children, and even in adults (3, 4, 7, 15). Also, a recent report has implicated RSV infection as a major cause of death in infants with congenital heart disease (N. E. McDonald, C. B. Hall, C. Alexson, and J. A. Manning, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 200, 1980). Since there are no useful RSV vaccines at present, the development of an effective antiviral agent may alleviate morbidity due to RSV in infants with congenital heart disease.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic nucleoside that has antiviral properties against a large number of both RNA and DNA viruses in vitro (2, 6, 7, 14, 16, 19, 22), has recently been shown to be inhibitory against RSV in vitro (5). The purpose of this study is to extend these observations to an in vivo model of RSV infection with the hope of developing an effective therapeutic drug.

Ribavirin was used by intraperitoneal injection and by aerosol to treat cotton rats (*Sigmodon hispidus*) infected intranasally with Long strain RSV. Effectiveness was assessed by measuring the effect of the drug on virus titers in the lungs and nasal turbinates of the animals, since the animals do not develop any clinical signs or mortality.

MATERIALS AND METHODS

Virus. The Long strain of RSV was obtained from R. M. Chanock (National Institutes of Health, Bethesda, Md.). Viral stocks were made in HEp-2 cells (Flow Laboratories, Inc.), and samples were frozen by immersion in a dry ice-ethanol mixture. The titer of these stocks was approximately 10^7 as determined by plaque assay (PFU per milliliter) and by tube dilution assay in tube cell cultures (50% tissue culture infectious doses per milliliter). Some experiments were done with RSV strain F520, which was passaged four times in cotton rat kidney and had a titer of 10^6 PFU/ml. F520 is an A_2 strain derived from F-059, a safety-tested RSV A_2 pool prepared by Louis Potash of Flow Laboratories.

Chemicals. Ribavirin (Virazole) was supplied by ICN Pharmaceuticals, Inc. The drug was dissolved in sterile phosphate-buffered saline at the stated concentration for administration by either the intraperitoneal or the aerosol route.

Assay for RSV. Virus titrations were performed by either tube dilution titration or plaque assay by testing serial 10-fold dilutions of virus-containing samples in Costar tissue culture dishes with a cluster of six wells (each 40 mm) by the method of Hruska et al. (5).

Animals. Cotton rats (*Sigmodon hispidus*) were obtained from the National Institutes of Health through the courtesy of R. M. Chanock and S. C. Suffin and bred at the University of Rochester or at the National Institutes of Health. Most studies used young cotton rats which were 4 to 6 weeks of age to insure that the virus titers would be uniform on the basis of age. However, animals as young as 10 days were used in the experiments measuring the effect of preloading the animals with ribavirin. In addition, all of the animals were randomly assigned to either control or treatment groups so that each animal had an equal number of litter mates. Animals were infected under methoxyflurane (Penthrane) anesthesia by intranasal instillation of 0.1 to 0.2 ml of tissue culture supernatant containing approximately 10^4 PFU of RSV. Animals were routinely sacrificed by cervical dislocation at 4 days after infection, when tissue levels of the virus were at their maximum. Lungs and turbinates were removed surgically, weighed, and homogenized in a Ten Broeck tissue grinder (Bellco Glass Inc.) in Eagle minimum essential medium. The suspension was divided into samples, frozen in a bath of dry ice and alcohol, and stored at −70°C until titration, usually 1 week later. A comparison was made between the titrations of homogenates before and after clarification (10 min at 1,000 × *g* in an International CR-5000 centrifuge).

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Since unclarified tissue suspensions gave a threefold-higher titer, all titrations were done without clarification, even though some toxicity was observed in the wells containing undiluted tissue suspensions.

Histology. A sample of lung was removed for histological examination from uninfected animals, RSV-infected animals, and ribavirin-treated infected animals. Samples were fixed in 10% buffered Formalin for 24 h, dehydrated in serial alcohol baths, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Aerosol administration. Aerosol was generated by a Solo-Sphere (McGaw Respiratory Therapy, Division of American Hospital Supply Corp.) nebulizer, which utilizes the Babington principle. The reservoir was refilled every 12 h with ribavirin solution. The nebulizer was operated at 20 lb/in^2 of pressurized air, with an aspirator setting of 60% and a flow rate of 5 liters/min. A 2 ft^3 (56-liter) tent was constructed around the cage and nebulizer by using a plastic bag. A specially constructed stainless-steel mesh floor was made for the cage to allow the feces and urine to fall to the bottom of the cage. No pine bark was used. The cage was cleaned daily when the nebulizer reservoir was refilled. The food bin was covered superiorly by aluminum foil in an attempt to prevent the drug from saturating the food.

The rate of consumption of the reservoir solution was 600 to 700 ml/23.5-h period, or approximately 0.4 to 0.5 ml/min.

Aerosol characterization. The concentration of ribavirin in the solution was measured by spectrophotometric analysis at a wavelength of 210 nm by using an extinction coefficient of 0.4 optical density unit per ml per μg per cm. The drug concentration in the aerosol was estimated by several methods. The aerosol was collected by precipitation in a cold "finger" cooled by dry ice-ethanol and quantitated by spectrophotometry, or the aerosol was dispersed into a 28-liter cylindrical glass jar and sampled onto glass-fiber filters (Gelman type A-E; Gelman Instrument Co.), which were also used to collect any radioactive isotopes when they were used. The filters were extracted with water, and the concentration of ribavirin was determined spectrophotometrically.

Deposition of aerosol. Nine cotton rats were exposed to aerosol from a nebulizer containing 1 g of ribavirin per liter; ^{59}Fe, at a carrier-to-iron concentration of 1 μg/ml, was used as a radioactive tracer to measure the drug concentration and the deposition in the lungs and nasal passages. Iron hydroxide at this concentration will not precipitate and, upon deposition in the respiratory tract, undergoes negligible absorption or translocation from the lungs over a period of many hours; additionally, at the concentration ratio used (ribavirin/iron of 1,000:1), there was no evidence of a complex formation or interference with the spectrophotometric assay. Hence, the drug-to-^{59}Fe ratio in the nebulizer solution prevailed in the aerosol, and the ^{59}Fe content of the lungs could be used to calculate ribavirin deposition. Groups of three animals were sacrificed at 30-min intervals. The animals were decapitated, and then the washed head and lungs were assayed in a NaI well counter by using an Ortec single channel analyzer (model 550) for measuring the 1.10- and 1.29-MeV photons. Additional samples were subjected to eight-stage impactor analyses of the aerodynamic particle size.

Tissue levels of ribavirin. An estimation of ribavirin tissue levels after intraperitoneal injection was made by using 5-^{3}H-labeled ribavirin (>98% pure) obtained from ICN Pharmaceuticals, Inc. Animals were injected intraperitoneally with 30 μCi of ribavirin dissolved in phosphate-buffered saline. Animals were sacrificed by cervical dislocation, and blood, lungs, spleens, and kidneys were removed, weighed, digested with Protosol, and counted in Biofluor by the procedures published by New England Nuclear Corp. The radioactivity of the tissue was determined in a Beckman LS-9000 scintillation counter and was adjusted for tissue quenching by using an external standard method (H-number method).

RESULTS

Cotton rat model of RSV infection. Cotton rats infected with RSV intranasally start to shed the virus 1 day after infection, reach maximum virus titers on day 4, and then show a rapid decline by day 7 (10). In this model of RSV infection, a neutralizing antibody to RSV is first detected between days 6 and 7, concomitant with the reduction in virus shedding. This model of RSV infection was used for the evaluation of ribavirin in vivo because of the convenient size of the animals, their low cost of maintenance, their uniform susceptibility to infection regardless of age, and their development of a desquamative, exudative rhinitis and proliferative bronchiolitis (10). In preliminary experiments, we confirmed the findings of Prince et al. (10) for virus shedding. By day 2 after infection, lungs and turbinates contained 10^2 50% tissue culture infectious doses per g of tissue. On day 3, these titers increased slightly, reaching a maximum of $10^{4.75}$ by day 4. On day 5, the titers started declining, and very low titers were present on day 7. In all subsequently described drug studies, the animals were sacrified at 4 days after infection to measure the effect of the drug on maximal tissue levels of virus.

Parenteral administration of ribavirin. Ribavirin administered by the intraperitoneal route inhibited RSV growth by approximately 90% when high doses were used (Table 1). No inhibition was seen when the drug was administered intraperitoneally at a dose of 40 mg/kg once daily starting 1 h postinfection. Although 80- and 120-mg/kg doses administered three times per day produced a significant reduction in either lung or turbinate titer, only 200 mg/kg given three times per day produced significant reductions of viral titers in both tissues.

Additional experiments were performed to determine the effect of preloading the animals with ribavirin intraperitoneally before infection or delaying the treatment for 24 h after infection. Animals were given ribavirin at a dose of 500

TABLE 1. Titers of RSV in tissues of cotton rats 4 days postinfection after intraperitoneal treatment with ribavirin

Treatment	Titer at dose of ribavirin given intraperitoneally (mg/kg):							
	40 q.d.[a] (n = 5)		80 t.i.d.[b] (n = 5)		120 t.i.d. (n = 5)		200 t.i.d. (n = 4)	
	Lungs	Turbinate	Lungs	Turbinate	Lungs	Turbinate	Lungs	Turbinate
Controls[c]	4.3 ± 0.71[d]	4.0 ± 0.6	4.3 ± 0.5	4.6 ± 0.4	5.0 ± 0.8	5.7 ± 0.6	5.6 ± 0.6	5.1 ± 0.5
Ribavirin	4.1 ± 0.45	4.5 ± 0.6	3.2 ± 1.1[e]	4.3 ± 0.6	4.1 ± 1.0	4.5 ± 0.7	4.5 ± 0.7	4.3 ± 0.6
P value[f]	<0.3	<0.1	<0.05	<0.2	<0.1	<0.005	<0.05	<0.05

[a] q.d., Every day.
[b] t.i.d., Three times a day.
[c] Controls were injected with phosphate-buffered saline intraperitoneally.
[d] Titer of virus in indicated tissues in $\log_{10}$ 50% tissue culture infectious doses per milliliter per gram of tissue.
[e] One of the five ribavirin-treated animals had no detectable virus in its lung samples, even though its turbinate sample was positive for virus.
[f] P was calculated by the Student t test.

mg/kg per day intraperitoneally once a day. Preloading with ribavirin for 3 days before infection inhibited RSV growth by 90% in both lung and nasal tissues ($P < 0.05$). However, when ribavirin was started 24 h after inoculation by the intraperitoneal route, little to no inhibition of virus growth was seen in lung and nasal tissues.

The lungs of cotton rats were also examined by light microscopy because of previous reports that RSV infection results in a proliferative bronchiolitis (10). However, we could not corroborate this previous report of lung pathology due to RSV. In three separate experiments, lungs were examined from sacrificed animals, which were either uninfected, infected, or infected and treated. No pathological changes could be detected in the lungs of control animals or in the lungs of infected or treated animals.

Animals tolerated ribavirin even at doses of 200 mg/kg three times per day and 500 mg/kg per day. There was no mortality associated with the administration of the drug, although one control animal which had received intraperitoneal saline died, probably from trauma due to the injection. Animal weights were recorded before and after the 4-day treatment. There was no appreciable difference between control and treated animals. Most animals either weighed the same or gained a few grams. However, no long-term toxicity studies were performed. Because ribavirin doses of 150 mg/kg or greater have been associated with weight loss and toxicity in other species of animals, we felt that these results, although inhibitory of the virus, indicated that intolerable amounts of the drug had to be used for treatment. Therefore, we measured tissue levels of the drug to determine whether the high drug doses were required because of poor distribution to the lungs after intraperitoneal injection.

Tissue levels of ribavirin were measured after intraperitoneal injection of 5-^{3}H-labeled ribavirin in 1.5 ml of phosphate-buffered saline. Maximum levels of tissue radioactivity were detected 1 to 3 h after injection. The distribution of radioactive compounds were as follows (in percentage of injected drug): liver, 25 to 33; kidney, 1.5 to 2; blood, 0.02 to 0.04; and lungs, 0.25 to 0.4. At an intraperitoneal dose of 200 mg/kg, a 50-g cotton rat would achieve a lung tissue level of approximately 25 to 40 μg per lung or 125 to 200 μg/g of tissue. However, it must be noted that total radioactivity measures both the active and the metabolized-inactive drug and, thus, does not represent the therapeutically active drug.

Aerosol administration of ribavirin. Because previous reports have found antiviral therapy administered by small-particle aerosol to be therapeutically more effective than other routes for the treatment of influenza in animal models (16–18), we tested the efficacy of aerosolized ribavirin against RSV infection in cotton rats.

Ribavirin administered by aerosol starting 1 h after infection exhibited an antiviral effect against RSV when the reservoir contained either 2 or 4 mg of ribavirin per ml (Table 2). Aerosol treatment with phosphate-buffered saline alone had no significant effect against RSV. Aerosol therapy with the nebulizer reservoir filled with ribavirin at a concentration of 1.0 mg/ml had no statistically significant effect on lung titers, but did reduce viral titers in the turbinate. Nevertheless, reassay of the samples by two different methods gave identical P values, although the plaque assay results were three to five times lower than the titer results measured by end-point dilution assay.

Characteristics of the aerosol. The Solo-Sphere nebulizer generated an aerosol the concentration of which was fairly constant over the whole time course between refills. Routinely, the nebulizer used 0.5 ml of reservoir fluid per min and needed to be refilled every 12 h. Table 3 shows the data generated in a mock run with the reservoir filled with approximately 1,000 μg of ribavirin per ml. The reservoir concentration increased only 14%

TABLE 2. Titers of RSV in tissues of cotton rats 4 days postinfection after continuous aerosol treatment with ribavirin

Treatment	Titer at concn of ribavirin in reservoir (mg/ml):							
	0 (n = 6)		1 (n = 6)		2 (n = 5)		4 (n = 10)	
	Lungs	Turbinate	Lungs	Turbinate	Lungs	Turbinate	Lungs	Turbinate
Controls[a]	5.0 ± 0.9[b]	5.4 ± 0.7	5.1 ± 0.7	4.9 ± 0.4	5.3 ± 0.5	5.75 ± 0.3	4.4 ± 0.6	4.35 ± 0.6
Ribavirin	4.5 ± 0.5	5.3 ± 0.3	4.4 ± 0.3	4.0 ± 0.7[c]	4.3 ± 0.5	4.8 ± 0.6	3.6 ± 0.1	3.6 ± 0.3
P[d]	<0.2	<0.35	<0.1	<0.025	<0.01	<0.01	<0.025	<0.0025

[a] Controls were not given aerosol treatment.
[b] Titer of virus in indicated tissues in $\log_{10}$ 50% tissue culture infectious doses per milliliter per gram of tissue.
[c] One of the six ribavirin-treated animals had no detectable virus in its turbinate sample, although its lung sample was positive for virus.
[d] P was calculated by the Student t test.

(from 960 to 1,092 μg/ml) over 12 h as measured spectrophotometrically. The aerosol was measured by collecting a 15-min timed specimen which had precipitated inside a cold finger. Although this method did not collect all of the specimen (typically it collected only 15 to 20% of the nebulizer output), it was reproducible, and it did give an estimation of the ribavirin in the output. Over a 12-h period, the concentration of drug collected in the cold finger increased only 7%. Thus, the Solo-Sphere nebulizer maintains an almost constant concentration of drug in both the reservoir and the aerosol over the 12-h operating period of the device. The aerosol particle size (mass median aerodynamic diameter) was measured by a seven-stage impactor. The mass median aerodynamic diameter was 1.12 μm, with a geometric standard deviation of 2.24. These parameters were derived from the aerosol droplet distribution (before evaporation), which had a mass median aerodynamic diameter of approximately 3 μm and a geometric standard deviation of 2.24.

The ribavirin concentration in a 28-liter chamber reached an equilibrium value of approximately 15 μg/liter of air after the nebulizer was operated for about 30 min with a reservoir concentration of 1 mg/ml as measured spectrophotometrically. Nine animals were placed in this chamber, and the pulmonary deposition of ribavirin was estimated by using an ^{59}Fe-labeled tag along with the ribavirin. Three animals were removed every 30 min, decapitated, and washed, and their heads and lungs were assayed for incorporated ^{59}Fe. The rate of accumulation in the total head is approximately 0.1 μg/min per animal (Table 4). No attempt was made to dissect out the turbinates. The rate of accumulation in the total lung was approximately 0.04 μg/min per animal or about 0.03 μg/min per g of lung tissue, which represents only about 0.01% of the nebulizer output. The amount of ribavirin deposited per gram of lung tissue in a 24-h period after aerosol treatment with a drug concentration of 4 mg/ml can be calculated by extrapolation to be about 173 μg or 5 mg/kg per day in a 50- to 70-g cotton rat.

DISCUSSION

This study examined the effect of ribavirin on experimental RSV infection in a cotton rat model (10). Previously, it had been shown that ribavirin inhibited RSV in vitro at concentrations which could be achieved clinically; i.e., concentrations between 3.0 and 10.0 μg/ml inhibited plaque formation of RSV by 50% or more (5). For this reason, the drug was tested in vivo to evaluate its possible clinical use.

The cotton rat model was chosen because it is the best detailed model in which infection can take place throughout the life of the animal and because viral titers of approximately 10^5 PFU per g of lung or nasal turbinates may be attained. Titers in tissues reach their maximum approxi-

TABLE 3. Characteristics of the aerosol with respect to volume in the Solo-Sphere nebulizer and time elapsed

Time (min)	Reservoir vol (ml)	No. of samples	Concn (μg/ml)		Ratio of aerosol to reservoir
			Aerosol[a]	Reservoir	
0–75	450	6	638 ± 73	960 ± 27	0.66
325	300	1		1,046	
650–700	100	4	682 ± 46	1,092 ± 14	0.63

[a] Aerosol was collected in a cold finger which was immersed in a dry ice-ethanol bath.

TABLE 4. Deposition of ribavirin in the tissues of cotton rats exposed to an aerosol from a nebulizer with a reservoir concentration of 1 mg of ribavirin per ml

Time (min)	No. of animals	Ribavirin deposition (μg per animal)[a]	
		Head	Lungs
30	3	3.5	1.4
60	3	7.0	1.9
90	3	9.5	3.2

[a] Estimated by measuring the tracer amount of ^{59}Fe deposited in the animals after exposure to an aerosol containing both ribavirin and ^{59}Fe.

mately 4 days after inoculation and rapidly diminish on days 5 and 6 concomitantly with the appearance of neutralizing serum antibody against RSV (10). Although the highest maximal virus production is observed in animals 14 days and younger, the older pups and adults produce virus to only slightly lower levels. In contrast, the other animal models are much more difficult to use in antiviral drug evaluations. For example, ferrets do not develop pulmonary infection past 28 days of age (11), primate models of RSV infection are too expensive and too scarce to employ in these studies (1, 12), and an inbred mouse model produces $^1/_{10}$ as much virus and is not as thoroughly characterized as is the cotton rat model (9). Although cotton rats do not die after infection, a previous report has mentioned the development of a mild bronchiolitis, with more extensive changes seen on days 4 through 6, characterized by ballooning epithelial cells and clusters of degenerating and dying eosinophilic cells (10), and with a return to normal histology by day 10. Ribavirin treatment of our animals was assessed by titers of viruses in the lungs and nasal turbinates and by the histology of lung tissue at day 4. We confirmed that virus production appears to be maximal in both lungs and turbinates on days 4 and 5; however, we were unable to demonstrate any bronchopneumonia or other pathological changes in the lungs of our infected animals either before or after ribavirin treatment. We do not know whether the inability to produce bronchopneumonia was due to differences in the virus strain or differences in the animals, even though we employed the same conditions as previously reported: the animals were from the same breeder colony, and the virus inocula were 10^4 PFU of Long strain RSV per animal. The effect of ribavirin on viral titers was assessed only on day 4 because serum antibody to RSV appears between days 6 and 7. Although it is possible that the reduction in tissue levels of RSV in these animals treated with ribavirin may have been due to a delay in replication of RSV by the drug, this is difficult to prove with this model because of the development of anti-RSV antibodies at times later than 4 days.

The only parameter which we found useful in evaluating the effectiveness of ribavirin in these studies was the reduction in viral levels in the tissues of cotton rats. Ribavirin administered both intraperitoneally and by aerosol was capable of reducing the titer of RSV in tissues by at least 90%. This reduction is most likely not due to a carry-over of ribavirin, since specimens were diluted to between 1/100 to 1/1,000 during the viral titration. At these dilutions, the ribavirin concentrations in the lung tissues would be below an inhibitory level. Only two animals treated with ribavirin had no detectable virus in one of their two tissue homogenates. However, these animals did exhibit a virus in their matched tissue homogenate (either lung or turbinate); therefore, the lack of a virus in these samples was most likely secondary to drug inhibition and not due to a failure of the animals to become infected with RSV. All other ribavirin-treated animals demonstrated only a reduction of the virus and not eradication. Virus titrations done both by plaque assay and by tube dilution assay demonstrated a significant inhibition of RSV growth by ribavirin, even though the endpoint dilution method would most likely not detect an effect unless it were large.

The significance of a 90% reduction in viral titer in lung and turbinate tissues in RSV infection is unknown, but when a reduction of a similar magnitude was observed after antiviral therapy of experimental influenza infection in mice, a significant reduction of mortality and changes in pulmonary inflammation were also observed (20, 21). Consequently, these results suggest that ribavirin may be therapeutic in RSV infection, but confirmation must await the development of an animal model which shows other objective symptoms of infection.

Previous reports have demonstrated the superiority of aerosol-administered antiviral therapy over intraperitoneal administration in a mouse model of influenza infection (17, 20). In contrast, this study showed that aerosol treatment with ribavirin reduced the amount of RSV in lungs and turbinates to about the same extent as intraperitoneal administration, although the aerosol route exposed animals to a smaller amount of the drug. The amount of virus reduction seen with aerosol treatment did not change appreciably, even when the drug concentration in the reservoir was quadrupled from 1 to 4 mg/ml. For that reason, no higher drug concentrations were tested, even though no adverse side effects were seen. It is possible that this plateau,

in effect, may be due to the characteristics of the nebulizer used in these experiments, and a comparison with the nebulizer used by others should be made (21).

The large doses of parenteral ribavirin required to treat RSV lung infection in cotton rats can possibly be explained by the pharmacokinetics of the drug. Less than 1% of the intraperitoneally injected ribavirin was found in lungs of cotton rats at 1 h as compared with 8% found in the lungs of common rats (Rattus species) given the drug orally. On the other hand, the concentrations of ribavirin found in livers of these two species were approximately the same (cotton rat, 33%, versus rat, 45%) (8, 13). Thus, it is possible that the reason that 5 to 15 times more drug was required in cotton rats is because lung levels are low due to either pharmacokinetics or liver metabolism. Although an antiviral effect was seen in RSV infections in cotton rats, parenteral administration with these high doses of ribavirin would probably be dangerous, since doses as low as 200 mg/kg per day are reported to cause emaciation, diarrhea, and death in common rats after 14 to 28 days. Aerosol delivery of the drug could circumvent the problem by directly delivering the drug to the diseased tissue without unnecessarily exposing other body tissues to deleterious concentrations of the drug. For aerosol, we calculated that an effective dose in the present experiments was 5 mg of ribavirin per kg of body weight per day. In addition, most of this ribavirin would be in the lung, although some would probably also diffuse into the bloodstream.

These experiments show that ribavirin is capable of inhibiting RSV in an animal model of infection and suggest that ribavirin may be an effective antiviral agent in naturally occurring human infection.

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